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Mutation screening of the 5-hydroxytryptamine$_7$ receptor gene among Finnish alcoholics and controls

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Abstract

Impaired central serotonin neurotransmission has been associated with increased aggression, impaired impulse control and diurnal activity rhythm disturbances among humans. Neuroanatomic distribution and pharmacological properties of the serotonin 5-HT$_7$ receptor suggest that it may play a role in psychiatric disorders and in circadian rhythm regulation. In this study a point mutation causing proline^{279} → leucine amino acid substitution in the 5-hydroxytryptamine$_7$ (5-HT$_7$) receptor gene was discovered. This 5-HT$_{7Leu279}$ variant was observed in six of 825 individuals, all of whom are heterozygous for the substitution. Three of them are alcoholic offenders (3/255), two are relatives of an offender without the 5-HT$_{7Leu279}$ allele (2/255) and one is a healthy control without any psychiatric diagnosis (1/248). The allele frequency of the 5-HT$_{7Leu279}$ variant is 0.004 (6/758) among Finns. Although the 5-HT$_{7Leu279}$ variant is approximately three times more common among alcoholic offenders than among healthy controls, it is not significantly associated with alcoholism or impulsivity in the present study. The 5-HT$_{7Leu279}$ allele may, however, be a predisposing allele in a subgroup of alcoholic offenders with multiple behavioral problems.

Keywords: Human; Receptors; Serotonin/genetics; Polymorphism; Impulsivity; Point mutation

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1. Introduction

Low brain serotonin activity has been associated with a variety of behavioral traits including impulsiveness and aggression, depression, suicide and alcoholism. Several studies have found a negative correlation between impulsive behaviors, aggression and concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of serotonin (5-hydroxytryptamine, 5-HT), in the cerebrospinal fluid (CSF) (Limson et al., 1991; Virkkunen et al., 1994a). Alcoholic, impulsive offenders with antisocial personality disorder have low mean CSF 5-HIAA concentrations (Virkkunen et al., 1994b). Although alcoholism, antisocial personality disorder and suicidality are seemingly heterogeneous and complex in their origin, they also appear to be strongly interrelated at underlying levels of causality. There are several family and adoption studies that indicate coinheritance of liability for alcoholism and antisocial personality disorder or antisocial behavioral traits (Cloninger et al., 1981; Cadoret et al., 1985; Linnoila et al., 1989). Studies on rhesus monkeys have shown that CSF concentration of 5-HIAA is heritable (Higley et al., 1993). Among rhesus monkeys, low CSF 5-HIAA is also correlated with increased physical aggressiveness (Higley et al., 1992). Therefore inherited differences in serotonin function could be a shared underlying vulnerability variable for an array of behavioral problems.

An approach to analyze causally complex genetically influenced traits is to directly evaluate the structure or function of known candidate genes, which play a postulated role in the implicated pathogenic mechanism. This strongly favors the detection of relatively rare alleles, which may be involved in the pathogenesis of a disorder. Among candidate genes for behavioral disorders with hypothesized serotonergic dysfunction are the enzymes controlling serotonin synthesis and metabolism, the serotonin transporter and serotonin receptors. In the present study we screened the coding sequence of the 5-HT_7 receptor gene for variants among Finnish alcoholic offenders and controls.

The 5-HT_7 receptor is a guanine nucleotide regulatory protein coupled receptor (GPCR). The human 5-HT_7 receptor gene is located on chromosome 10q23.3-q24.3, and it consists of three exons (Gelernter et al., 1995; Erdmann et al., 1996). On the basis of its sequence, it is thought to have the typical GPCR structure of seven hydrophobic transmembrane helices separated by three extracellular and three intracellular loops (Bard et al., 1993). Pharmacologically the 5-HT_7 receptor resembles serotonin receptors in the 5-HT_1 and 5-HT_2 families. Unlike these receptors which inhibit adenylate cyclase activity, activation of the 5-HT_7 receptor stimulates adenylate cyclase. In this regard, the 5-HT_7 receptor resembles the 5-HT_4 and 5-HT_6 receptors. There are at least four 5-HT_7 receptor isoforms that are produced by alternative splicing (Heidmann et al., 1997), but the differences in their pharmacological profiles are not yet known. Although studies to clarify the physiological role of the 5-HT_7 receptor are limited, it has been suggested that 5-HT_7 receptors are involved in the regulation of circadian rhythms (Lovenberg et al., 1993). Circadian rhythms in mammals are controlled by the suprachiasmatic nucleus of the hypothalamus, and pharmacological studies suggest that the 5-HT_7 receptor may modulate circadian rhythms (Lovenberg et al., 1993). Alcoholic offenders with intermittent explosive disorder often have a desynchronized diurnal activity rhythm which includes increased activity during the night (Virkkunen et al., 1994b). The 5-HT_7 receptor also has a distinct pharmacological profile, which includes a high affinity for clozapine and related atypical antipsychotic agents as well as for some typical antipsychotic agents (Roth et al., 1994). Thus, the 5-HT_7 receptor may play a role in the therapeutic action of these drugs, and it may be involved in the pathophysiology of certain psychiatric disorders. These could include antisocial personality disorder of the impulsive and explosive type, which is associated with disturbances of diurnal rhythm.

2. Methods

2.1. Subjects and psychiatric diagnosis

A detailed description of the subjects and the

study protocol is presented elsewhere (Virkkunen et al., 1994b). Briefly, subjects included in the study are alcoholic offenders who were ordered to undergo forensic psychiatric examination after committing a violent crime. The controls are a random sample of volunteers of socio-economic background similar to the alcoholics. We have also included relatives of the alcoholics and controls in the present study. The same forensic research psychiatrist interviewed all subjects. Two other psychiatrists using the DSM-III-R criteria blind-rated the diagnoses. All subjects gave a written consent, and the study protocol was approved in Finland by the Department of Psychiatry and the Helsinki University Central Hospital Institutional Review Boards. In the US, the protocol was approved by the National Institute of Mental Health Institutional Review Board and the Office for Protection from Research Risks.

Samples of other Caucasians are 67 unrelated Centre d'Étude du Polymorphism Humain parents who were used as another comparison population.

2.2. Screening of the 5-HT_7 receptor

The coding sequence of the 5-HT_7 receptor was partly screened for polymorphisms by single strand conformational polymorphism (SSCP) analysis. The initial screening was done using 21-mer primers amplifying the fragment between 633–1216 bp of the published 5-HT_7 receptor cDNA sequence (Bard et al., 1993) and cutting the long fragment with different restriction enzymes. The PCR reaction mix contained 100 ng genomic DNA isolated from immortalized cell lines, 0.8 mM dNTPs, 0.5 μM primers, 0.15 mM [α-^{33}P]dCTP, 0.25 U of AmpliTaq (Perkin Elmer Cetus, Norwalk, CT), 60 mM Tris–HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$ and 1.5 mM $MgCl_2$ in a total volume of 5 μl. Samples were amplified using a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles consisting of 30 s at each of three temperatures: 95°C, 55°C and 72°C. After amplification, a solution containing 95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromphenol blue were added for a total volume of 25 μl. After denaturation at 95°C for 3 min, 3.5 μl of the mixture was loaded on a MDE® Gel (AT Biochem, Malvern, PA) and electrophoresis was performed at room temperature for 16 h at 4 W. The gel was dried and Kodak XAR film was exposed for 12 h at room temperature. The PCR products were sequenced by direct thermal cycle dideoxy sequencing (CircumVent®, New England Biolabs, Beverly, MA). One polymorphic allele was found, C(836) → T(836), and new 21-mer primers were designed which amplified a 302-bp DNA fragment, which corresponded to nucleotides 633–935.

2.3. 5-HT_7 receptor genotyping

Because the restriction enzyme XhoI recognizes the C(836) DNA sequence yielding two fragments (230 bp and 72 bp) but leaves the T(836) DNA sequence uncut (302 bp), the XhoI site was used in a PCR-restriction fragment length polymorphism (RFLP) analysis. Fragments were separated by electrophoresis in 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

To rapidly screen large numbers of samples, we used allele-specific amplification (ASA). For ASA, the PCR reaction was run with an allele-specific upper primer (5′-ACAAGTTTCCTGGCTTCCT-3′) and a lower primer recognizing both alleles (5′-GCTGCGATAGGTGGTCCTCAGGT-3′). The annealing temperature was 68°C. PCR products were separated on a 7.5% polyacrylamide gel and the appearance of a band at 351 bp was considered to be evidence of a positive allele-specific reaction. All positive samples were confirmed by RFLP analysis, which also detected heterozygosity.

2.4. Statistical analysis

Fisher's exact test with two-tailed probability was used in the statistical analyses for association.

3. Results

We discovered an amino acid substitution in

Table 1
Frequencies of 5-HT_7 genotypes and the Leu^{279} allele among Finnish psychiatrically interviewed unrelated controls, alcoholic offenders and their relatives. All offenders had a diagnosis of either alcohol abuse or alcohol dependence. As a comparison population, frequency of the 5-HT_7 Leu^{279} allele was also determined in another Caucasian population

Subject category	Pro^{279}/Pro^{279}	Pro^{279}/Leu^{279}	Leu^{279} allele frequency
Control	247	1	0.002
Alcoholic offender	252	3	0.006[a]
Relative	253	2[b]	0.004
Total (Finns)	752	6	0.004
Other Caucasians	67	0	0.000[c]

[a] Fisher's exact test $P = 0.62$ vs. controls.
[b] See Table 2.
[c] Fisher's exact test $P = 1.00$ vs. Finns.

the 5-HT_7 gene using the SSCP method. The amino acid substitution proline$^{279} \rightarrow$ leucine is due to a cytosine(836) to thymidine DNA base substitution (Fig. 1). More efficient RFLP and ASA methods were subsequently developed to screen a large number of individuals for this rare polymorphism. The allele frequency of this polymorphism is low (0.004 in all Finns genotyped) and all the subjects with the Leu^{279} variant are heterozygous (Table 1). The detailed characteristics of the individuals with Leu^{279} are presented in Table 2. Three of them were alcoholic offenders, two were relatives of an offender without the Leu^{279} allele and one was a healthy control. Among the three alcoholic offenders several biochemical variables, including concentrations of CSF 5-HIAA, homovanillic acid, 3-methoxy-4-hydroxyphenylglycol and concentrations of CSF hormones (testosterone, arginine vasopressin and adrenocorticotrophin) and day/night urinary cortisol excretion, were determined. None of these values clearly differed from the values of alcoholic offenders without the polymorphism (data not shown). Leu^{279} allele did not associate significantly to alcoholism ($P = 0.31$ vs. controls), impulsivity ($P = 0.50$ vs. controls), or violent behavior with alcohol abuse or dependence ($P = 0.62$ vs. controls). However, all three of the alcoholic offenders with the Leu^{279} allele fulfilled the criteria of multiple psychiatric diagnoses according to the DSM-III-R. They also showed a clear trait of habitual violence. Also the two subjects with the Leu^{279} allele who are relatives of one offender without the Leu^{279} allele had psychiatric problems (Table 2). The mother had made a suicide attempt and the brother fulfilled diagnostic criteria for alcohol abuse. The control carrying the Leu^{279} allele has not had any problems related to alcoholism or violent behavior.

Table 2
Characteristics of six subjects who were Pro^{279}/Leu^{279} heterozygotes at the 5-HT_7 receptor gene (diagnoses were blind-rated according to DSM-III-R criteria)

Sex	Age (years)	Subject category	Diagnoses	Note
Male	21	Alcoholic offender	AD, drug and cannabis abuse, BP, ASP	Alcoholic father and mother, suicide attempts
Male	44	Alcoholic offender	AD, BP, narcissistic personality, ASP	Alcoholic father and mother
Male	37	Alcoholic offender	AD, IE, schizoid	Alcoholic father, suicide attempts
Female	49	Relative	—	Mother of 2581 (alcoholic offender), suicide attempt
Male	30	Relative	—	Brother of 2581 (alcoholic offender), alcohol abuse
Male	24	Control	—	Student

Note: AD, alcohol dependence; BP, borderline personality; ASP, antisocial behavior; IE, intermittent explosive (DSM-III).

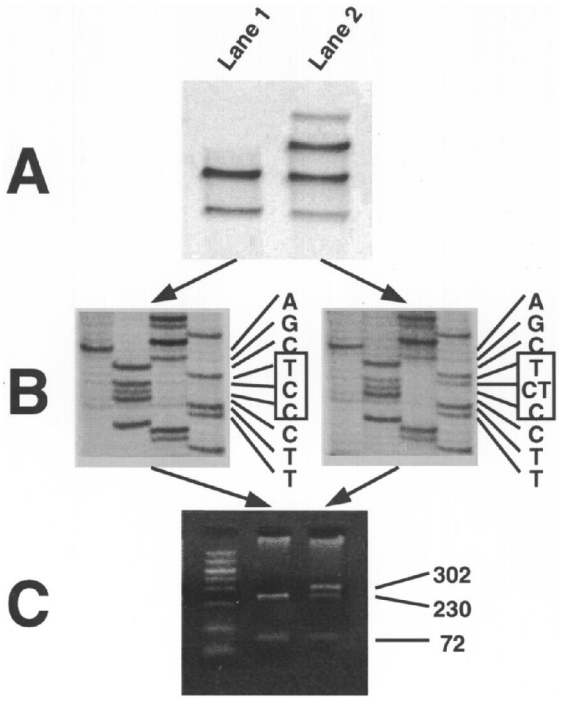

Fig. 1. SSCP (A), sequencing (B) and RFLP (C) gels for the homozygous Pro^{279}/Pro^{279} genotype (Lane 1) and the heterozygous Pro^{279}/Leu^{279} genotype (Lane 2).

4. Discussion

In the present study we detected the Leu^{279} variant, a naturally occurring amino acid substitution, in the sequence of the 5-HT_7 receptor gene. Leu^{279} is a rare allele and it does not show a significant association to alcoholism or impulsivity among Finnish alcoholic offenders. The Leu^{279} allele is, however, approximately three times more common among the alcoholic offenders than among controls and may represent a predisposing allele in a subgroup of alcoholic offenders with a combination of antisocial personality disorder and multiple personality disorders. Also the two other subjects with the Leu^{279} variant, who were relatives and could not be used in the association analysis, had psychiatric problems (see Table 2). Although a large number of subjects were included in the study, the low allele frequency of the Leu^{279} variant limits the statistical power of the association analysis. Direct analysis of the function of the variant 5-HT_7 receptor in these subjects is in progress and eventually will clarify the biological significance of the variant receptor.

The $Pro^{279} \rightarrow Leu$ substitution occurs in the putative third intracellular loop of the 5-HT_7 receptor protein. Although the third intracellular loop exhibits the highest diversity within the family of homologous GPCRs, structure–function analyses of several GPCRs have suggested that the third intracellular loop has specific regions which bind various G-protein subunits and also contains phosphorylation sites (Franke et al., 1988; O'Dowd et al., 1988; Varrault et al., 1994; Oksenberg et al., 1995). A computer-assisted modeling study on the hamster β_2 adrenergic receptor, which is also positively coupled to adenylate cyclase, suggests that there are, in addition to the seven transmembrane segments, two shorter segments within the third intracellular loop that could form amphiphilic helices (MaloneyHuss and Lybrand, 1992). The interaction of ligand induces conformational shifts and reorientation of these helices of the third intracellular loop to activate the G protein (MaloneyHuss and Lybrand, 1992). The amino acid residue Pro^{279} is located at the C-terminal end of a stretch of residues that is invariant across all three primates sequenced as well as in the rat (Fig. 2). Thus, it is highly plausible, but not proven, that this Pro^{279} residue terminates a conserved helical segment positioned at the intracellular end of the fifth transmembrane segment. Modeling of the secondary structure of the 5-HT_7 receptor protein (Genetics Computer Group, version 7, Madison, WI) predicts that the Leu^{279} substitution results in a decrease in hydrophilicity and an increase in likelihood for helix formation (data not shown). We therefore postulate that the most likely consequence of the $Pro^{279} \rightarrow Leu$ point substitution is a change in local protein structure, which would affect G-protein coupling. Such an alteration at the molecular level could be sufficient to modify the function of the 5-HT_7 receptor. This hypothesis can be examined by expressing the two 5-HT_7 alleles in a cell line and comparing their functional properties.

The results described in this article were presented in preliminary abstract form 1995 (Pesonen

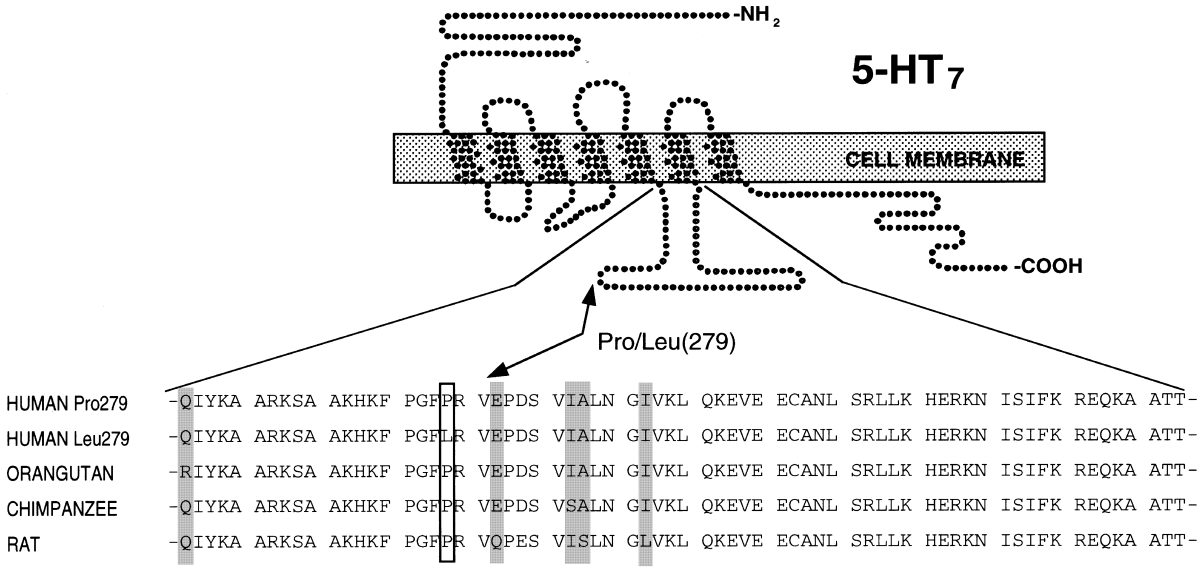

Fig. 2. Schematic representation of the molecular structure of the 5-HT_7 receptor. The arrow indicates the site of the $Pro^{279} \rightarrow Leu$ substitution within the third intracellular loop. Also shown is the alignment of the third intracellular loop of 5-HT_7 between the human Pro^{279} (Bard et al., 1993) allele, the human Leu^{279} allele, the homologous sequences of the orangutan, the chimpanzee and the rat (Shen et al., 1993). The amino acids, which vary across the four species, are shaded and the conserved Pro^{279} site, the location of Leu^{279} substitution in some humans, is boxed.

et al., 1995). Since then there has been a published 5-HT_7 receptor screening study on patients with schizophrenia and bipolar affective disorder (Erdmann et al., 1996). The investigators of that study were able to find an additional non-conservative polymorphism, $Thr^{92} \rightarrow Lys$, and one silent nucleotide substitution, which were both in a gene region that we did not screen. These variants were also very rare and did not associate to the risk of developing schizophrenia or bipolar affective disorder.

In the present study, we were able to screen less than half of the coding region of the 5-HT_7 receptor, since the intron-exon boundaries of the gene were not available at that time. The region we screened covers the fragment between the putative fourth and seventh transmembrane helices. We were not able to discover any additional polymorphisms among Finnish alcoholic offenders in this area. Although the Leu^{279} variant is rare and only six heterozygotes were detected, it is a potentially important naturally occurring variant of the serotonin 5-HT_7 receptor.

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